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Biomonitoring role of some cellular markers during heat stress-induced changes in highly representative fresh water mollusc, *Bellamya bengalensis*: Implication in climate change and biological adaptation

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ABSTRACT

Owing to increasing concern of global climate-change, temperature rise is of great interest which can be primarily evaluated from the seasonal variations in some organisms. Aquatic environment can be extremely stressful to its inhabitants because most of them are poikilothermous. In the present study, attempt was made to evaluate the biological effects of oxidative-stress and adaptive/antioxidant capacities during temperature variations (36–40 °C for 24hrs to 72hrs) in *Bellamya bengalensis* both in environmental and laboratory conditions by testing some biomarkers like HSP70, catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH) and glutathione reductase (GR). The biomarker potency of the molecules and the anti-oxidative metabolic-network was postulated and extrapolated to find its resemblance to the climate-change associated organismal variations. In a natural and eco-restored environment in the Eastern part of India, 10–20 fold increases in CAT, SOD and HSP70 protein expressions (Western blot results) were noticed in *Bellamya* paralleling to their increased enzymatic activities (gel zymogram studies) due to the seasonal (summer versus winter) temperature variation. It is evident from the consecutive three years' study that this variation resulted in the unfavorable physico-chemical changes of water quality parameters like dissolved oxygen, biochemical oxygen demand, alkalinity and consequently decreased the animal density in summer. And that was revived due to their higher reproduction-rate in post rainy/winter season when temperature normalizes resulting in a restoration of favorable environment. In laboratory condition, the reduced GR and increased GPx indicated the oxidative damage as evident by higher tissue MDA level following to higher mortality. Changes in SOD and CAT activities suggest activation of physiological mechanism to scavenge the ROS produced during heat stress. However, when mortality increased at different time points (36 °C − 72 h and 38 °C − 72 h), these enzyme activities also decreased as they failed to save the tissues from ROS. The results suggest that temperature variation does alter the active oxygen metabolism by modulating antioxidant enzyme activities, which can be used as biomarker to detect sub-lethal effects of climate change-associated pollution. The parity in environmental and laboratory experimental results may justify this laboratory experiment as model heat-stress experiment and indicate temperature as a universal stressor which alone or in combination with other water parameters initiates a consistent adapting behavior. The *Bellamya bengalensis* being the highest faunal representative in its habitat may serve as a good bioindicator species.

1. Introduction

Global warming is expected to have devastating effects on fresh-water wetland ecosystems. The living-system of poikilotherms experience daily and seasonal temperature variation which has biological consequences (Jonsson and Jonsson, 2009). Temperature can change water-quality parameters like pH, alkalinity, DO, BOD, COD and other meteorological variables (Vaghefi et al., 2015). Adverse situations tended to stress from molecular oxygen resulting in cellular damages which require the cells to re-direct its resources towards maintenance.

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Previous and recent reports suggest that consumption of O_2 by poikilotherms can be related to rapid temperature changes that would influence the metabolic regulations of such organisms (Newell, 1966; Lofquist et al., 2016).

Organisms have evolved a variety of strategies to respond to external or internal environmental challenges by metabolic changes in order to maintain cellular homeostasis (Mizrahi et al., 2014). The organisms living at their physiological stress limit will be affected by the continuous rise in temperature and only those endowed with sufficient defense mechanisms will be able to survive (Fabbri et al., 2008). Now it is to enquire whether the adaptive strategies during the seasonal temperature variations (which are cyclical but drastically of wide range) have some resemblance with the sustained but obvious unidirectional temperature increase generated by global warming mediated climate-change. And it is also to evaluate that how long living body could be able to withstand this slow but unidirectional temperature changes (Bernardo and Spotila, 2006). Indeed, this is important to evaluate the biochemical impacts on organism generated by the alterations of physicochemical variables led by temperature during seasonal changes. The adaptive factors and the stress markers have a great role to translate the environmental physicochemical changes into metabolic and biochemical changes in the living systems (Chukwuka et al., 2014) and thereby to assign the status of bioindicator species (Jamil, 2000).

Ecosystems with a high degree of biodiversity can cope with more temperature mediated stress than those with less biodiversity due to the reciprocity and counterbalancing nature of the members inhabiting in that ecosystem (Wood et al., 2017). Stress responses after being measured range from those at the subcellular and biochemical levels to those at the ecosystem level. Eventually, the stress withstanding ability is consequently deemed among the strongest forces of natural selection (Fischer et al., 2010).

Reactive oxygen species (ROS) are continually generated as consequences of normal metabolic pathways. However, generation and degradation of ROS levels are controlled by delicate cellular control mechanisms (Halliwell and Gutteridge, 2007). Oxidative stress is caused by an imbalance between the generation of intra- and extracellular ROS and the ability of the antioxidants to scavenge those free-radicals (Lushchak, 2011). Exposure to heat causes the production of potent oxidants and free radicals capable of damaging important cell components such as proteins and DNA. Recent findings reveal that glutathione-s-transferase gene was significantly induced in internal and external tissues of Manila clam, *Ruditapes philippinarum*. Other genes like manganese and copper-zinc superoxide dismutase (MnSOD and CuZnSOD) were also induced in this species (Umasuthan et al., 2012). This suggests that the cellular adaptation might be initiated by the antioxidants and some drug metabolizing enzymes. In addition to the GST, few more abiotic factors have also been shown to induce cholinesterase in some marine molluscs like *Mytilus galloprovincialis*, *Nucella lapillus* and *Monodonta lineate* (Tim-Tim et al., 2009). In response, the cell initiates antioxidant enzyme systems and produces free radical scavengers like non protein thioles and GSH (Doyotte et al., 1997; Chainy et al., 2016). Antioxidant enzymes are some of the most common biomarkers used in environmental monitoring (Regoli et al., 1998; Prego-Faraldo et al., 2016). The enzymes usually respond rapidly and sensitively to biologically active pollutants (Fitzpatrick et al., 1997; Sandrini-Neto et al., 2016). Glutathione (GSH) is a low molecular weight scavenger of oxygen radicals (Regoli et al., 1998) and an electron donor for the antioxidant enzyme GPx, which increases rapidly in cells exposed to elevated temperatures (Verlecar et al., 2007). Glutathione (GSH) is often used in biomarker studies, as it is an overall modulator of cellular homeostasis (Ringwood et al., 1999; Khan and Ringwood, 2016). On the other hand, MDA, being obvious stress/free-radical markers, can be linked to the mortality of the organism (Köprücü et al., 2010). The cellular stress response can be elicited from a wide range of stressors, which include extreme temperature, heavy metals, ultraviolet light, gases, hypoxia, hyperoxia, and exposure to alcohols (Voellmy et al., 1985; Abele et al., 2011). Heat shock protein 70 (HSP70) has been identified as potential biomarkers for environmental stress in fishes, molluscs and almost all organisms (Wepener et al., 2005; Liu and Chen, 2013; Dutta et al., 2014; da da da Silva Cantinha et al., 2017a, 2017b).

The objective of the current study was to screen some dependable biomarkers as a measure of heat-induced stress. *Bellamya bengalensis* (Lamark 1882), a highly representative fresh water molluscan gastropod was selected to evaluate the impact of the temperature stress in natural environmental as well as laboratory condition. The stress protecting chaperones like Hsps also up regulated to protect themselves in response to stress (Dutta et al., 2014). Temperature-fluctuation has been shown to be related with the life span of *Caenorhabditis elegans*, ***a free living soil inhabiting nematode*** via HSP70 expression (Galbadage and Hartman, 2008). Population density and reproduction potentiality of gastropod have been found to be with stress withstanding ability (Raut, 1981).

The present study has attempted to establish the use of suitable biomarkers in a bioindicator fresh water molluscan species in order to address the impact of global warming. In addition, it was also intended to provide information about the maintenance of population and adaptive mechanism of the same species, *Bellamya bengalensis* to overcome seasonal temperature variations in natural environment with ongoing impact of climate change.

2. Materials and methods

2.1. Selection of study site, collection and treatment of studied faunal component

Adult specimens of *Bellamya bengalensis,* were collected from an ecorestored and un-interferred wetland having an area of 40200 m^2 and maximum depth of 5 m, located at Gurguripal about 10 km away from Midnapore town of South-Western part of West Bengal, India (22.432° N, 87.218°E) during winter (December -January) when water temperature was about 15 °C − 17 °C. However, their life cycle and population density were monitored in this wetland throughout the year. Heat stress experiments were done in the University laboratory.

This species was selected for its easy availability and demand as high protein food. After the collection from the natural environment, specimens were kept in plastic trays in the laboratory (temperature 18 °C −19 °C) with adequate water and food (leaves of spinach, lettuce and green algae) for acclimatization.

To study the effect of temperature, a fixed number of individuals (Nos. 20) of *Bellamya* were kept in plastic trays and the trays were placed in the incubator having fixed temperature (36 °C, 38 °C and 40 °C) for varied periods (24 h, 48 h and 72 h). Mortality was monitored during this heat stress experiment. After treatment, the respective alive animals were collected and dissected to collect digestive glands for biochemical and histological studies.

2.2. Assessment of the Population density and biomass of Bellamya bengalensis

The snails were collected once in a month from July, 2011 to June, 2014 from wetland by hand picking or by a hand net dragging over the vegetation. From this wetland, three sub samples were collected from each sub-sampling sites (East, West, North, South) covering an area of 1 m^2 along a transect. Therefore, all total 12 sub samples were collected in each month for both of the study sites. Average of those samples was presented as density in Fig. 1. Several molluscan species viz. *Bellamya bengalensis, Pila globosa, Lymnaea luteola, Thiara tuberculata, Indoplanorbis exustus* were recorded from the study site. Dominance of species was ascertained on the basis of relative abundance using Brockmann-Jerosch scale. Because of its higher abundance during all months, *Bellamya bengalensis* was selected for further study of heat

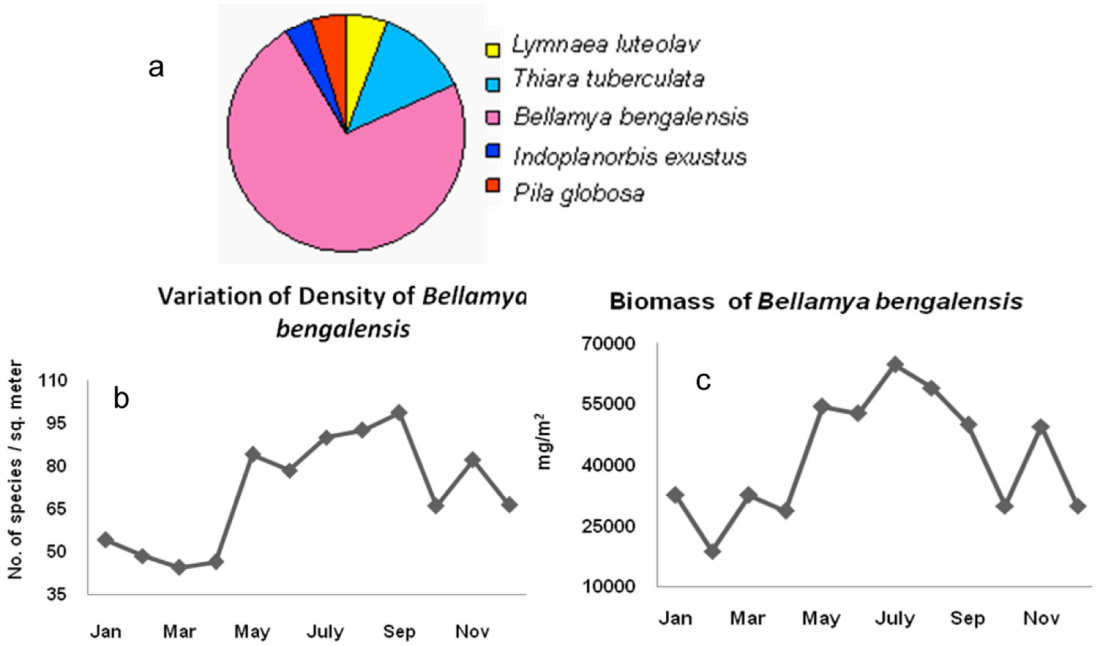

Fig. 1. Relative abundance of *Bellamya bengalensis* in the water body (month July). A greater representation of *B. bengalensis* justifies its utility in environmental stress related studies (a). Relative species density evaluated throughout the year (b) and biomass density of *Bellamya bengalensis* was evaluated throughout the year. The highest biomass of *B. bengalensis* was noticed in the third quarter (Jul-Sep) of the year (c).

stress experimentation. The body length of the experimental test animal was measured to the nearest 25–35 mm and weight of snail was taken to the nearest 2 mg. The biomass was calculated by drying the snail in an oven of 90 °C until constant weight achieved.

2.3. Preparation of cytosol for Western Blot and determination of oxidative stress

After collection, glands were homogenized in Lysis buffer containing 50mMTris-Hcl, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM $NaOV_4$, (v/v) $NP_{40,}$ 1 mM PMSF and centrifuged at 10,000 rpm for 30 min at 4 °C. Supernatants were utilized for protein measurement (Bradford, 1976). These were kept in aliquot at − 20 °C until use. Proteins (20–50 µg) from cytosolic fraction were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were incubated overnight at 4 °C with respective primary antibodies after blocking with 5% bovine serum albumin (BSA). Interspecies cross reactive antibodies against Manganese Superoxide Dismutase (MnSOD) (cat. no. 611580, BD-Pharmingen) and Hsp70 (cat. No. 610607, BD Pharmingen, against amino acids 429–640 of human Hsp70) were used in this investigation. The β-Actin antibody was purchased from Imgenex (Cat. no. IMG-5142A). The membranes were incubated with 1:5000 dilutions of the appropriate peroxidase-conjugated secondary antibodies and/or alkaline phosphatase-conjugated antibodies and developed for detection by chemiluminescence or colorimetry. Western blots were scanned and densiometric analysis of the scans was performed by using Image J software.

Catalase activity was measured spectrophotometrically by calculating the rate of degradation of H_2O_2 the substrate of enzyme (Aebi, 1984). Superoxide dismutase (SOD) activity assay was performed by in-gel assay that is combining of polyacrylamide gel electrophoresis and densitometry of negative banding (Chen and Pan, 1996). Reduced Glutathione (GSH) was estimated as initially described by Ellman (1959) and modified according to the method of Davila et al. (1991). Glutathione peroxidase (GPx) activity was measured according to Levander et al. (1983). Glutathione reductase (GR) activity was measured according to Carlberg and Mannervik (1985). MDA level was monitored according to following the method of Davila et al. (1991).

2.4. Tissue preparation for histology

For Light microscopic study, heat stressed animal's tissues (36 °C, 38 °C, and 40 °C in 24, 48 and 72 h) were collected and processed for histological staining. After taken out from treated *Bellamya*, digestive glands were kept in Bouin's fluid for 1 h. After that, glands were kept in serially in Xylene (for 2 min), Xylene: Paraffin (3:1, 30 min), Xylene: Paraffin (1:1, 30 min), Xylene: Paraffin (1:3, 30 min) and in full Paraffin for 3 h. After that, block became ready for sectioning. Sections were cut in a microtome at < 6 µm breadth and slides were prepared. Eosin-Haematoxylin counter stains were used for the staining of the tissue sections.

2.5. Analysis of physicochemical parameters of water

The water samples were collected at monthly intervals throughout a year with the help of indigenously deigned water sampler from different sampling sites of study area. Collected water was analyzed by following standard methods (APHA, 2005; Trivedy and Goel, 1984).

The statistical analyses were done by using the SPSS for Windows statistical software package (SPSS Inc., Chicago, IL, USA, 2001). Correlation statistics were done to test the association of the mortality rate and different biochemical parameters. P value < 0.05 is considered statistically significant.

3. Results

Temperature was found to be positively correlated with alkalinity, BOD and COD of a natural and eco-restored water body (Table 1). The DO has been shown to be negatively correlated with the ambient temperature. The DO is noticed to be positively correlated with the life-density (Table 1). The mortality rate has been found to be positively correlated with Hsp70, Catalase, Catalase activity and SOD activity and showed a relationship at the 1% level of significance while revealed significant positive relationship with GPx at the 0.1% level of significance (Table 1). The mortality rate was found to exhibit significant

Table 1
Effect of seasonal temperature variation on physicochemical properties of water. Correlation between physicochemical variables and density/ Mortality of *Bellamya bengalensis*.

Parameters	Relation with	Correlation coefficient
Temperature	Alkalinity	0.671***
	DO	− 0.443**
	BOD	0.448**
	COD	0.646***
Density	Alkalinity	− 0.369*
	pH	0.348*
	DO	0.390*
Mortality Rate	MDA	0.917***
	GPx	0.979***
	GR	0.947***
	Catalase activity	0.896**
	SOD-activity	0.960**
	Catalase expression	0.849*
	Hsp70 expression	0.877**

Results of Pearson's correlation analysis among different natural physico-chemical parameters of the water sample, density through 36 months (3 years / 9 seasons) and experimental mortality-rate vs some biochemical-antioxidant parameters. The numerical values denote correlation coefficient (r).

* Significance at 5% level.

** significance at 1% level and.

*** significance at 0.1% level.

negative correlation with GR activity at the 0.1% level of significance (Table 1). It was noticed that at more stressful condition (for higher temperature and/or longer duration), the animal started to die. In present study, GPx activity was found to be correlated with Hsp 70, Catalase, Catalase activity, SOD and GR activity (Table 1). GR exhibited significant negative correlation with Hsp 70, Catalase activity and SOD activity (Table 1).

Relative abundance of *Bellamya bengalensis* in the water body (month July) suggest its density to be ~79% (Fig. 1a). Relative species and biomass density were also higher in this month (1b and 1c respectively). Mortality rate was found very consistently increasing with the increase of exposure in experimental temperature. Increase in mortality paralleled with the oxidative-stress increase (MDA) in the animals (Fig. 2).

Antioxidant enzyme MnSOD was found to increase by 2.5 fold at 36 °C for 48 h and 4 fold at 38 °C for 72 h (Fig. 3a). However, catalase increased by ~50% at 36–38 °C heat treatment for 72 h ($p < 0.01$). The increasing response of GPx ($p < 0.05$ to $p < 0.01$, Fig. 3c) and decreasing GR activity (Fig. 3d) have been noticed in this study. The GR activities decreased significantly (~50% in 36–38 °C for 72 h; $p < 0.01$) in response to increasing heat exposure.

In the present study, the intracellular GSH initially increased moderately (20% in 38 °C for 24 h $p < 0.01$) with the increase of temperature and exposure time (Fig. 4a) and then decreased. This basically agrees with the GPx and GR data. Catalase levels increased by almost 5 fold when *Bellamya* was subjected to heat shock of 36 °C-72 h and this elevated level of catalase was further augmented (~7.5 folds) on exposure to higher temperature of 38 °C-72hrs. MnSOD expression was enhanced to ~5 fold in the temperature of 36 °C after an exposure of 48 h and ~ 6 fold in 38 °C-72hrs exposure of temperature. After exposure in 40 °C-24 h, MnSOD was increased upto 3 fold (Fig. 4b).

Seasonal temperature variation was found to be highly effective in augmenting the adaptive responses in *Bellamya* (Fig. 5). In summer, the animal mortality rate was increased and the expression of protein biomarker catalase, SOD and HSP70 was found to increase 5–15 fold compared to that of winter season. The activity of SOD, catalase have also found to increase in parity with the increase of temperature (Fig. 5).

Studies of histological changes on different temperature stressed individuals under the species, *Bellamya bengalensis* in comparison to the same species under control state have revealed different modifications of cellular organizations. The glandular cells of the digestive tract of gastropod mollusc, the control (untreated) and temperature stressed (36 °C-24 h, 36 °C-48 h 36 °C-72 h 38 °C-24 h 38 °C-48 h 38 °C-72 h 40 °C-24 h) specimens of *Bellamya bengalensis* have undergone histological changes at the cellular levels which are being depicted in the plates (Fig. 6). The different heat and time exposure tended to increase the necrotic tissue lesion which resulted the disintegration of the lining of the basal/basement membrane. Most of the cells in control were matured and mononucleated. Few of the dividing cells have been seen to be bi- or multi-nucleated. The excretory vacuoles and secretory granules are regularly present in control cells with a certain limits in number but on increased temperature exposure, numbers of random shaped vacuoles and deep stained secretory granules have been observed to increase in number. It has been found that as a result of elevated temperature, the smoothness of the lining of the lumen were completely impaired and completely degenerated and tissue debris materials gradually covered the open space of the lumen making its circumference very small, suggesting the necrotic degeneration of the lining.

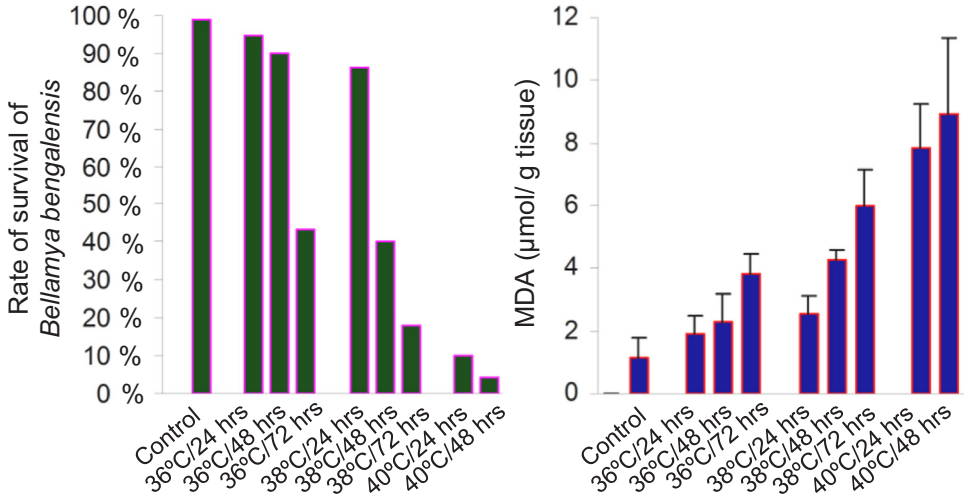

Fig. 2. Oxidative Stress marker (MDA) was found positively correlated with the Mortality rate. Mortality rate was found very consistently increasing with the increase of exposure in experimental temperature. Increase in mortality paralleled with the oxidative-stress increase (MDA) in the animals.

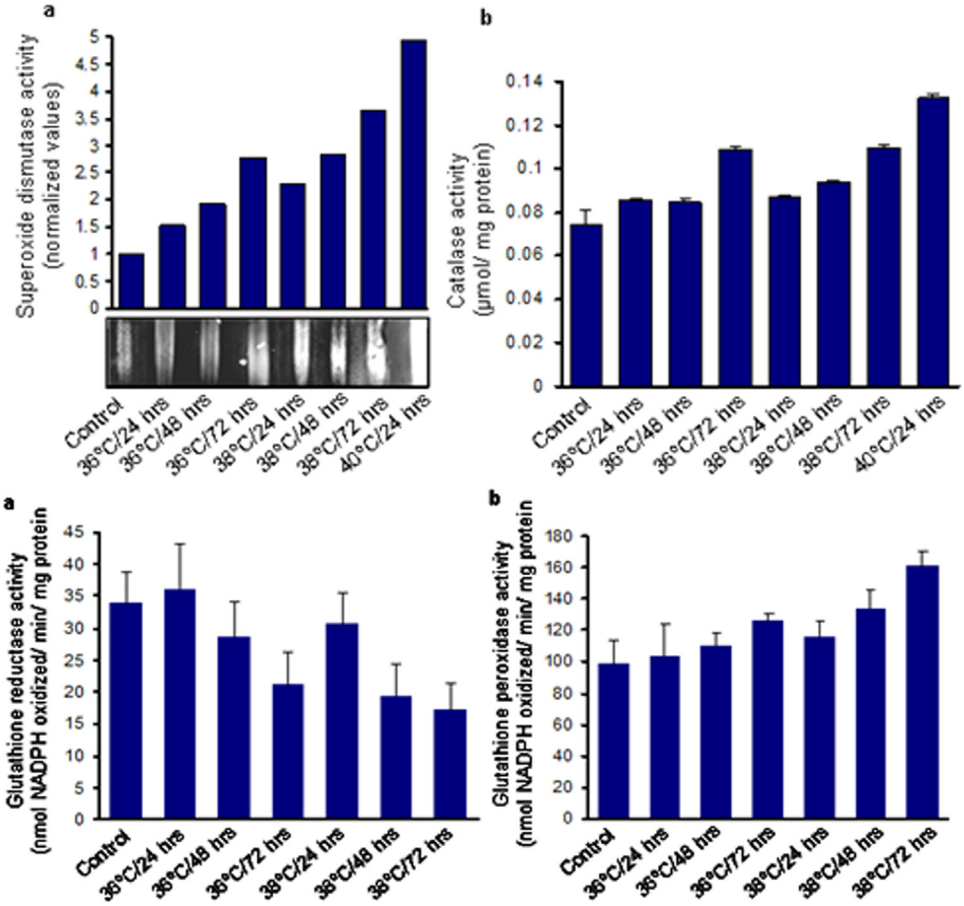

Fig. 3. Effects of heat stress are shown on a. SOD b. Catalase c. GR and d. GPx. Temperature drastically tended to induce SOD activity and appreciably increase catalase activity in the experimental organism. Decrease in GR activity and increase in GPx activity suggest a potential depletion of antioxidant capacity in the test organism in response to heat stress.

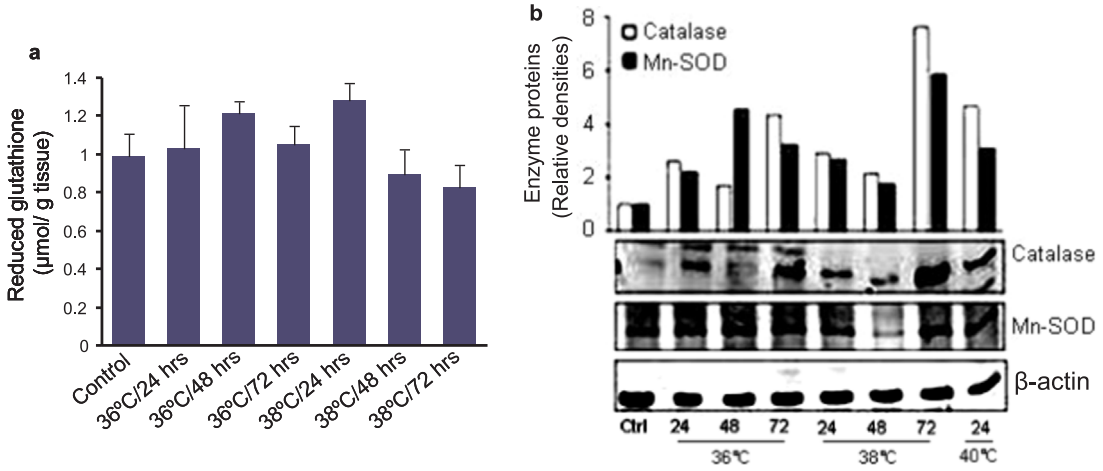

Fig. 4. a. Regulation of Glutathione (GSH) metabolism and b. Expression of Catalase and SOD are shown in this figure. GSH level initially increased in an adaptive responses and then it remained unaltered in higher temperature suggesting a toxicity responses. The Western blot of Mn-SOD and catalase suggest appreciable increase in response to heat stress.

4. Discussion

The current study explores some suitable and dependable stress biomarkers in *Bellamya bengalensis*, a highly representative gastropod mollusca in a natural fresh water aquatic ecosystem. Biological effects due to seasonal temperature variations and associated physicochemical changes in natural conditions have been studied and linked with the results from laboratory experimental analysis. However, in the wider perspective, the systematic extrapolation of our present findings on the correlation between two experimental conditions will help for the better understanding of the climate-change and global-warming related adaptive responses in the biological systems. Nevertheless, the population dynamics and interrelation amongst different living systems are important for a particular species to maintain their life process in a habitat. An investigation in the similar type of water body like the present experimental site, suggests that a varied number of zooplankton communities i.e. rotifera, cladocera, copepoda, ostracoda, protozoa and certain insect larva inhabit in this system (Pradhan and Chakraborty, 2006). The differential rate of occurrences of adults and juveniles of *Bellamya* in different seasons are thought to be due to the reason of

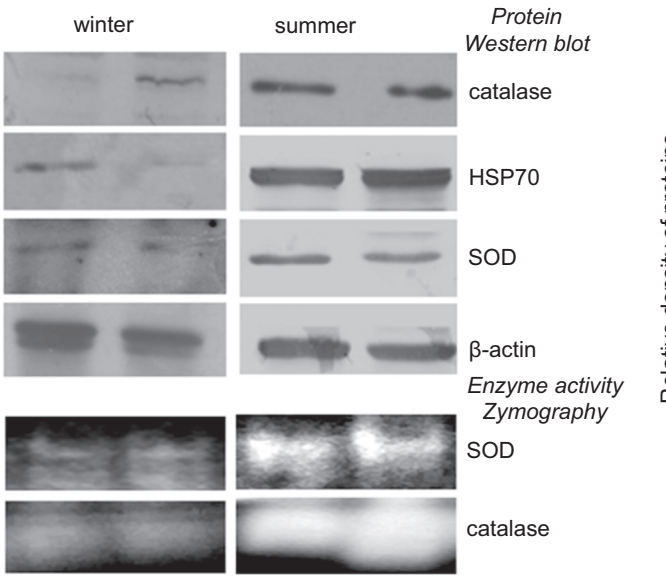

Fig. 5. Seasonal (summer/ winter) temperature induced expression of Catalase, SOD and HSP70 in *Bellamya bengalensis*. Seasonal temperature variation was found to be highly effective in augmenting the adaptive responses in *Bellamya*. The figure suggests that the activity of SOD, and catalase were also increased in parity with the increase of temperature.

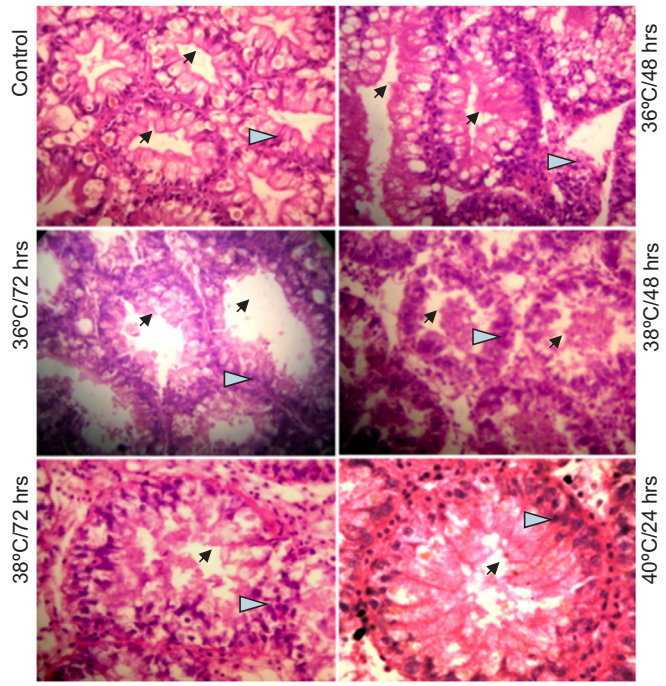

Fig. 6. The histological views of the transverse section of digestive glands of *Bellamya bengalensis* of control state and heat stressed (36 °C, 38 °C-48/72hrs, 40 °C- 24 h) are shown by Haematoxylin-Eosin staining (magnification x 40). The figure indicates certain change in the glandular cells of the digestive tract of gastropod mollusc exposed to higher temperature with comparison to that of control (black arrowhead). Few of the dividing cells have been seen here to be bi- or multi-nucleated suggesting a higher rate of pyknosis (colored arrowhead).

conducive environmental set up. The variations in mortality and reproduction rate maintain the steady state of the population size of a species and its proportion in the whole living community (Fig. 1). Natural stress related decrease in *Bellamya* density during and post summer period is counterbalanced by their higher reproduction ability during the post rainy season. It is evident from the present study that *Bellamya* experiences an appreciable seasonal temperature-change resulting in changes in its different biochemical parameters including adaptive responses. This may not be able to protect the animal completely from the higher chances of mortality. Nevertheless, helps in maintaining compatible reproducibility by the adapted animals. During rainy season, the water-volume/flow increases and the concentrations of several soluble and particulate matters decrease. This large volume of water experiences autochthonous and the allochthonus supply of nutrients which makes the water-body suitable for flourishing phytoplanktons and zooplanktons (Pradhan and Chakraborty, 2006). These are the good sources of nutrients for the newly hatched juveniles of *Bellamya*. Moreover, dilution of water reduces its toxicity and promotes juvenile growth. Inversely, low quality of concentrated water during summer renders higher toxic-threats encountered by the animals (Sharma, 2011). The periodicity in the change of water quality and mortality- reproduction rate, keep the animal density in a dynamic state. But the climate change due to global warming related temperature change is a slow, steady but unidirectional changes, the impact of which on biological system has not well been characterized.

Molluscs require sufficient levels of cataions like Ca^{2+}, Mg^{2+} and anions like bicarbonates as well as dissolved oxygen (DO) for their normal growth (Richardson et al., 2004). Fluctuation of these ingredients interfere animal density. The antioxidant genes and genes involved in Ca^{2+} signaling and homeostasis are found to be over expressed in *Laternula elliptica*, an Antarctic bivalve. Heat stress has been shown to respond in increasing protein turnover and folding, intracellular signaling and trafficking and cytoskeletal activity (Truebano et al., 2010). As a result of major perturbations like increase of temperature and physico-chemical variations in water quality, the population declines significantly due to increase in toxicity and/or decrease in essential nutrients/materials in the water (Mackie and Claudi, 2009). In the current study, the temperature showed a direct influence on the level of DO of the water body (Table 1). Proper availability of O_2 maintains the balance of several metabolic processes and energy expenditure in the body. The influence of mitochondrial respiratory chain and oxygen consumption has been linked to the higher temperature adaptation in marine intertidal molluscs, such as oysters. This study has explained the interactive role of natural contaminant metals and temperature during different metabolic processes (Sokolova, 2004). In the present study, it is noticed that the seasonal variations altered the DO and some other physicochemical factors in water in summer and post-rainy/winter seasons (Table 1). The environmental temperature versus animal density has been shown to be negatively regulated with the DO (Table 1) indicating the limited access of electron transport chain with sufficient O_2. Oxygen limitation contributes to oxidative stress and other impairments of metabolic processes. During the thermal tolerance, both toxic manifestation and adaptive responses takes place from systemic to cellular to molecular levels (Pörtner, 2002). Dissolved

oxygen (DO) has been shown to be related to different physicochemical properties of the water body. It was found that a significant correlation existed between temperature and other chemical factors in the present study. This correlation (Table 1) has an impact on toxicity pattern and surveillance of animals in the present study. The possible interactive roles of temperature and other aquatic physico-chemical properties influenced the HSP70 and anti-oxidant proteins for their higher expression to withstand the heat and related stress in the animal (Figs. 4 and 5). Studies on stress-induced HSP70 regulations in *Bellamya* are very scanty. Evidence suggests that the physico-chemical variables like heavy metal (cadmium) can induce this protein in *Biomphalaria glabrata* even at its sub-lethal dose suggesting HSPs role as a dependable biomarker during environmental stress. Moreover, this induction augments the acquired tolerance in this mollusc during stressful situation (da Silva *et al.* 2017).

The pattern of adaptive responses in *Bellamya* in laboratory experiment (exposed to different temperatures for varied duration) suggest that heat alone or in combination with other environmental factors (seasonal study) exerts similar types of effects on HSP and antioxidant functions. The ubiquitous role of HSPs as shown in some snail and other organisms suggest its overall role in the resistance against a number of stressors. And this is due to its highly interactive metabolic capability in all most all organisms (Mizrahi et al., 2017). The unique nature of these stress markers indicate that temperature can be regarded as a universal stress factor. Indeed, heat and salinity may be regarded as the most ancient stressors which are shown to participate in several biological phenomena. But report reveals that heat, not the saline stress, influenced the growth of shallow-marine bivalve mollusc shells of *Phacosoma japonicum* highlighting the universality of heat as the unique stress factor (Schöne et al., 2003).

When the environmental perspective is concerned, it is clearly evident that HSP70 exerts its most important role in the possible adaptive mechanism (Fig. 5). A large number of stressors like anoxia, ischaemia, toxins, protein degradation, hypoxia, acidosis and microbial damage may induce HSPs including HSP70. The fundamental role of HSPs in the regulation of protein- synthesis, protein folding/targeting and kinetic partitioning between folding makes them very dependable regulators within the cell (Roberts et al., 2010).

Owing to temperature fluctuation and repeated variation in the seasonal changes, catalase, SOD and HSP70 protein expression significantly increased paralleling with the increase in concerned enzymatic activities (Fig. 5). Report focuses on HSPs regulations in response to *daf-2* gene functions via insulin-like growth factor signaling pathway which is responsible for the longer life span and growth of the *C. elegans* in higher temperature (Pörtner, 2002). This suggests that not only as a chaperonin protein, HSPs also tend to exert its influence on other gene expressions. Environmental stressors may promote HSPs regulations and its further impact on other genes' expression like CAT, SOD and CYP4Y1 in the generation of specific cellular protective responses (Rossi et al., 2016).

In addition to HSP70, the GRP78 (glucose-regulated protein, 78 kDa a related HSP70 family member) was significantly up-regulated and exerted their adaptive responses in evolutionary divergent antarctic marine molluscs (*Laternula elliptica* and *Nacella concinna*), a bivalve and a gastropod, respectively (Clark et al., 2008). This suggests stress can influence the glucose homeostasis and energy metabolism for better adaptive responses via HSPs. Several extended role of HSP70 has been documented. It has been detected in the neuroendocrine system development, and reproductive process of mollusc indicating higher reproducibility in post rainy season, after exposure from summer stress as evident in the current study (Fig. 1). Furthermore, the induction of HSPs was found to be related to the phosphorylation of stress-regulated p38 mitogen-activated protein kinase (p38 MAPK) and cJun-N-terminal kinases (JNKs) in molluscs (Liu and Chen, 2013). This suggests that heat stress may regulate the expression of several transcriptional factors and selected gene expressions.

Several environmental-factors are individually and synergistically effective to interfere the biological system. The diversity and the population density of the animal have been influenced by the habitat availability (Hastie et al., 2003). The young animals were found to be comparatively more protective against hypoxic and hyperoxic conditions due to the adaptive role of elevated SOD, GPx, GR and catalase (Amicarelli et al., 1997) in the protection of their macromolecules like DNA and proteins (Vosloo et al., 2013). The water temperature had significant effects on SOD activity and protein oxidation of gills during post monsoon in the mussels (Di Salvatore et al., 2013). Report reveals that the temperature increase from a lower point up to 11 °C resulted in a significant decrease of SOD activity but with this change, malondialdehyde (MDA) concentration was found to increase compared to that of controls at 0 °C, suggesting lower temperature can play little role in protecting due to hypometabolic state in the organism, but less adaptive due to a lack of a scope of pre-acclimatization (Donelson et al., 2011). In higher temperature, metabolic rate is increased with increased O_2 consumption and oxygen-radicals generation in the tissues. In environmental scenario, low DO at increased temperature limits the O_2 consumption by the organism adding a compounding effects of hypoxia associated stress. The adjustments of mitochondrial densities and their functional properties appear as a critical process in defining and shifting thermal tolerance windows. The finding of oxygen limited thermal tolerance owing to loss of aerobic scope is in line with Taylor's and Weibel's concept of symmorphosis, which implies that excess capacity of any component of the oxygen delivery system, is disadvantageous (Pörtner, 2002).

In the present experimental study, it has been noticed that little higher the basal temperature level can initiate certain degree of pre-acclimatized state promoting to increase in different antioxidant enzymes and HSP70 during further increase in temperature. But, whether the animal experienced a pre- or post-adaptive state, beyond its ultimate stress withstanding ability, it faces a higher rate of mortality. It is evident from the current experimental data. Notwithstanding, in environmental context, this pre-acclimatization due to slow increase of ambient temperature in summer enabled the animal to prepare for a steady state reproduction at rainy season that potentially compensate loss of animals from the stress-related mortality.

Acute heat stress is supposed to decrease cell viability, lysosome membrane stability, double- and single-stranded DNA breakage in both native and invasive mussels, *Mytilus californianus* and *Mytilus galloprovincialis* (Yao and Somero, 2012). Reactive oxygen species and oxidative stress have been demonstrated to damage cellular macromolecules (Schieber and Chandel, 2014). Interfered lysosomal membrane stability and necrotic tissue damage may be the result of heat stress and toxicity from transition metals like copper in *Mytilus galloprovincialis* (Lam.) (Negri et al., 2013) that varies during seasonal variation. The result from the present study on the alterations of the antioxidative properties in the *Bellamya bengalensis* after the heat stress justifies the cellular toxicity which resulted in the temperature dependant tissue damage (Fig. 6) and animal mortality (Table 1 and Fig. 2). A temperature decrease usually induces an ordering effect in membrane phospholipids that can lead to membrane dysfunction. Ectotherms typically counteract this temperature effect by remodeling membrane lipids as stipulated in the homeoviscous adaptation theory (Dubousquet et al., 2016). Pacific oyster *Crassostrea gigas* exposed to acute thermal stress showed increase in the expression of cytochrome P450 and multidrug resistance (MDR1)) and regulation of the cell cycle (p53) (Farcy et al., 2009). The expressions of enzymes like SOD, CAT and GST, and genes like mt-10 and mt-20 showed a substantial increased pattern in animals exposed for 24 h to heat stress compared to that of control (18 °C). But the co-expression in the presence of metal contaminant like Ni caused a pronounced increase in lipid variations (Banni et al., 2014).

Glutathione is regarded as the cellular protectant. In the cases of GSH depletion, cellular system experiences oxidative stress (Davila et al., 1991). So the mortality rate increases significantly. The increase

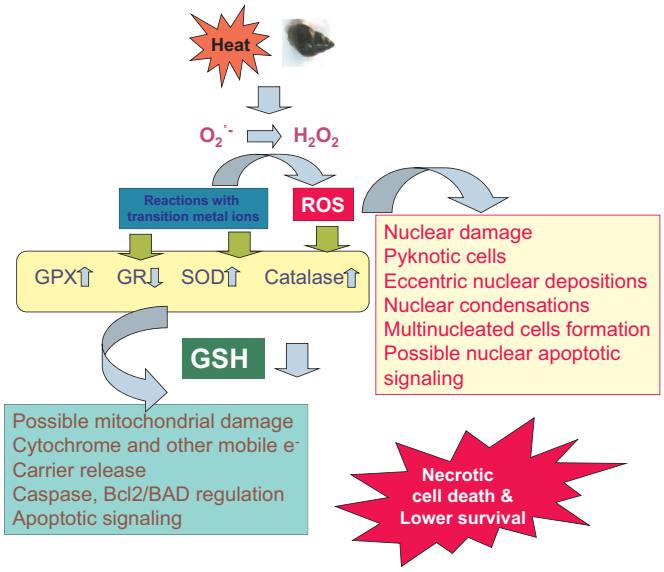

Fig. 7. A brief diagrammatic representation of heat induced oxidative stress in *Bellamya* and its impact on several important cellular antioxidant components. Severe impairments of these components result in necrotic and degenerative cell death. High load of oxidative stress results in significant tissue degenerations and higher mortality of the organisms.

in GPx activity results in the greater utilization of GSH content of the cells in our laboratory investigation (Fig. 3). This utilization is neither compensated by GR-mediated slavage pathway nor by the de novo synthesis of GSH which is evident from final low level of cellular GSH. This indicates the occurrence of higher H_2O_2 in the cells. The H_2O_2 is nullified from the cells by GPx activity with the help of GSH (Doyotte et al., 1997). Indeed, the depletion of GSH restricts its utility in other protective metabolic processes and finally increases the animal mortality (Fig. 2). In the current study, it is noticed that the increase of catalase activity in *Bellamya* was not sufficient to withstand the heat stress due to a permanent depletion of GSH after its long-term exposure. The inactivation of different types of free radicals by SOD and catalase is noticed in all exposure schedules in the current study. The irreversible tissue damage as noticed in the histoarchiecture is caused by the intense oxidative threats noticed by the higher MDA level. Indeed, the necrotic membrane damage, pyknotic cell formation, nuclear condensation, multinucleated nuclei generation as noticed, are the initial stage of cellular apoptosis. These are the main cause of cell damage and increase in animal mortality.

The different heat and time exposure tended to increase the necrotic tissue lesion, the outcome of which are characterized by the disintegration of the lining of the basal/basement membrane. Most of the cells in control are matured and mononucleated. Few of the dividing cells are binucleated. The excretory vacuoles and secretory granules are regularly present in control cells with a certain limits in number but on increased temperature exposure, numbers of random shaped vacuoles and deep stained secretory granules have been observed to increase in number. Those granules are migrated far away from the lining of the lumen and deposited at the layer of the connective tissues of the intracellular region or glands. In extreme high temperature (38^0C) along with time of exposure (72 h), the intracellular connective tissues were tended to be completely dissociated and the shapes of the cells were completely not recognizable (Fig. 6). Present results are summarized in Fig. 7 which briefly explains the source of reactive oxygen species and possible anti-oxidative responses initiated in the cells. When the ability of protective functions fails, animals face drastic oxidative / necrotic tissue damage and finally enter in a death phase.

5. Conclusions

So, it may be hypothesized that heat can exert stress alone or in association with some physico-chemical variables in a consistent pattern. And the synergistic effects of those factors are magnified in the course of time and the degree of their exposure. However, after a certain time period, when the adaptability of the organism against stress become exhausted, the animal finally face the mortality. It may be suggested that the thermal stress originated from the possible global warming and climate change might help in the interpretation on the role of different cellular organelle and indicate some biomolecules as biomarkers in aquatic pollution monitoring programmes. The sustenance of the stress initiates a situation in the organism so that it becomes incapable to enhance its stress withstanding capacity. This is likely to be occurring during the condition of global warming. In case of seasonal variations, where the animals are accustomed by their circadian rhythm to the cyclical temperature alterations, that is almost ineffective in case of irreversible climate-change situation. So, to survive in this situation, the temporary acclimatization mechanism may not be effective and animals may need a long term evolutionary adaptive mechanism.

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Conflict of Interests

None

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